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Signalling, cell cycle and pluripotency in embryonic stem cells

Tom Burdon, Austin Smith and Pierre Savatier

Pluripotent mouse embryonic stem (ES) cells can be expanded in large numbers *in vitro* owing to a process of symmetrical self-renewal. Self-renewal entails proliferation with a concomitant suppression of differentiation. Here we describe how the cytokine leukaemia inhibitory factor (LIF) sustains self-renewal through activation of the transcription factor STAT3, and how two other signals – extracellular-signal-related kinase (ERK) and phosphatidylinositol-3-OH kinase (PI3K) – can influence differentiation and propagation, respectively. We relate these observations to the unusual cell-cycle properties of ES cells and speculate on the role of the cell cycle in maintaining pluripotency.

Mouse embryonic stem (ES) cells are the *in vitro* counterparts of an *in vivo* population of cells, known as the epiblast, that are specific to the early embryo [1–3]. Epiblast cells are pluripotent, which means that an individual cell can give rise to all cell types of the foetus. ES cells retain the developmental identity and potential of the epiblast even after prolonged culture. This has been shown conclusively by their complete integration into a developing embryo after being reintroduced into the blastocyst [4]. ES cells can efficiently colonize the germ line, resulting in chimaeric animals. These produce functional gametes, which allows ES cells to be used as vehicles for introducing sophisticated genetic modifications into mice [5]. ES cells can also undergo multilineage differentiation *in vitro* and produce a range of well-differentiated progeny [6,7]. Currently there is considerable interest in the prospect of exploiting this potential in analogous human pluripotent cells [8] to generate specific, differentiated types of cell for drug development, for therapies based on cell replacement, and for delivering gene therapies.

Less attention has been paid to the unusual proliferative properties of ES cells [9–11]. ES cells are derived without the intervention of any immortalizing agent, do not undergo either crisis or senescence, and retain a diploid karyotype. They proliferate without apparent limit [12] and can readily be propagated

clonally. They can multiply in the absence of serum and are not subject to contact inhibition or anchorage dependence. In fact there is no known means of inducing cell-cycle arrest and quiescence in ES cells. Apart from the normal karyotype, these are features that are typical of transformed cells and, indeed, ES cells are tumorigenic. In contrast to their behaviour when introduced into the early embryo, they produce teratocarcinomas when injected into adult mice. Thus ES cells can be considered as conditional tumour cells.

Embryonic stem cells undergo symmetrical self-renewal – that is, they produce two identical stem cell daughters when they divide. Self-renewal entails the suppression of differentiation during proliferation. Here we review current data on the regulation of ES cell self-renewal by signalling networks and discuss the relationship between cell-cycle control and the retention of pluripotency.

Cytokine-dependent activation of STAT3 drives ES cell self-renewal

The propagation of mouse ES cells is dependent on the presence of leukaemia inhibitory factor (LIF) or related cytokines that can activate signal transduction from cell-surface receptors [13–15]. LIF can be provided by a feeder layer of embryonic fibroblasts [16,17] and/or as a recombinant protein. LIF engages a heterodimeric receptor complex consisting of two related cytokine receptors, LIF receptor (LIFR) and gp130 [18]. This complex activates associated Janus-associated (JAK) tyrosine kinases that phosphorylate the receptor chains. The phosphorylated tyrosines then act as docking sites for proteins containing Src homology 2 (SH2) domains that might themselves be phosphorylated by the JAKs (Fig. 1).

The signal transducer and activator of transcription (STAT) family of transcription factors

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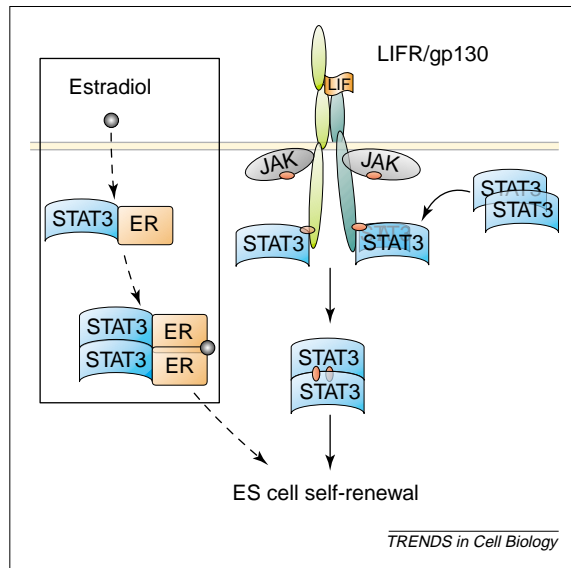


Fig. 1. LIF-dependent activation of STAT3 blocks ES cell differentiation and promotes self-renewal. Leukaemia inhibitory factor (LIF) stabilizes the association of LIFR and gp130 cytokine receptors. Resultant activation of receptor-associated JAK kinases causes the recruitment, tyrosine phosphorylation and dimerization of STAT3. The STAT3 dimers then translocate to the nucleus, where they control the transcription of genes regulating self-renewal. The importance of STAT3 is confirmed by the demonstration that a conditionally regulated form of the transcription factor STAT3ER can, when activated, substitute for LIF addition (boxed insert). Abbreviation: ER, ligand binding and dimerization domain of the oestrogen receptor.

bind receptor phosphotyrosines and are key substrates for JAKs. Phosphorylation of STATs promotes their dimerization through reciprocal interactions between an SH2 domain and phosphotyrosine. This triggers their translocation to the nucleus and their binding to target sites on DNA. In ES cells, LIF predominantly activates STAT3 [19].

Recruitment and activation of STAT3 is essential for self-renewal of ES cells [19,20], and expression of an inhibitory STAT3 mutant in ES cells forces differentiation [19,21]. Studies using a chimaeric STAT3 molecule that can be activated directly by estradiol (Fig. 1) indicate that STAT3 activation is not only necessary but might be sufficient to block differentiation [22]. Activation of this chimaeric molecule sustains ES cell self-renewal without the addition of LIF. It should be noted, however, that these experiments were carried out at moderate to high densities of cells in the presence of serum, which might provide additional signals that support ES cell viability and/or proliferation [22].

ERKs antagonize ES cell self-renewal

Signalling downstream of gp130 is not limited to activation of STAT3 but includes stimulation of the Ras/mitogen-activated protein kinase (MAPK) pathway. The ERK MAPKs p42 and p44 regulate many different cellular responses in somatic cells and have particularly well-documented roles in proliferation and differentiation. In its simplest

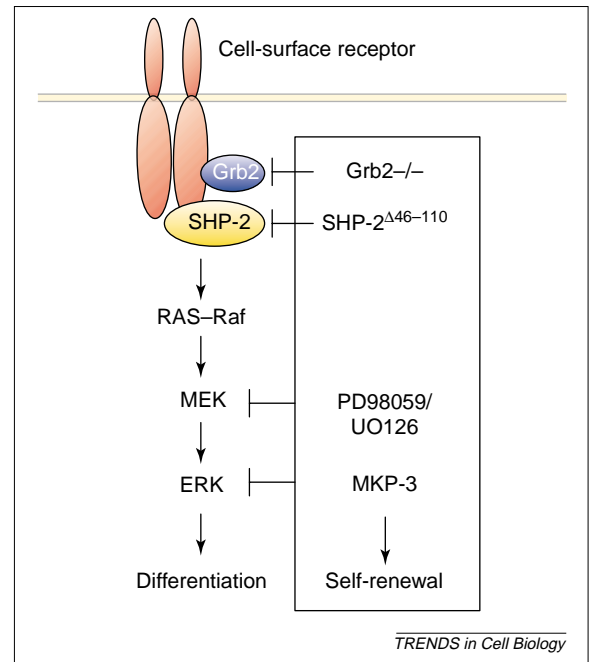


Fig. 2. Reduction in Ras/MEK/ERK signalling promotes ES cell self-renewal. Binding of the adaptors Grb2 or SHP-2 couples cell-surface receptors to the Ras/Raf/MEK/ERK signalling pathway. Interference with this pathway by mutation of *Grb2* or *Shp2*, by inhibiting the activation of MEKs with the small-molecule inhibitors PD98059 and UO126, or by dephosphorylating ERKs by mitogen activated protein kinase phosphatase 3 (MKP-3), limits the differentiation of ES cells and promotes self-renewal.

form, the ERK pathway is engaged through the recruitment of a complex containing the Grb2 adaptor and Sos guanine-nucleotide-exchange factor to activated receptors. Localization of Sos at the membrane promotes activation of Ras. This initiates a cascade of transphosphorylations involving Raf and MAPK kinase (MEK) kinases that culminates in activation of ERK [23] (Fig. 2). Active ERKs phosphorylate cytoplasmic targets and also undergo nuclear translocation, which enables them to modulate the activities of transcriptional regulators such as Elk, Ets, Myc and the serum response factor (SRF).

Receptor recruitment of the Grb2–Sos complex can be indirect. In the context of LIFR–gp130, a key intermediate in this recruitment is the protein tyrosine phosphatase SHP-2. Tyrosine phosphorylation of SHP-2 generates binding sites for Grb2. SHP-2 also associates with the scaffold protein Grb2-associated binder protein 1 (Gab1). This protein recruits the lipid kinase PI3K and, by binding the resulting phospholipid products through its N-terminal pleckstrin homology (PH) domain, stabilizes the association of the SHP-2–Gab1–Grb2 complex at the membrane and potentiates coupling to Ras [24].

In ES cells, eliminating the SHP-2 binding site from a chimaeric gp130 receptor blocks coupling to the Ras pathway but enhances the self-renewal response [25]. This effect is partly due to the elimination of a negative feedback effect on JAK activity [26],

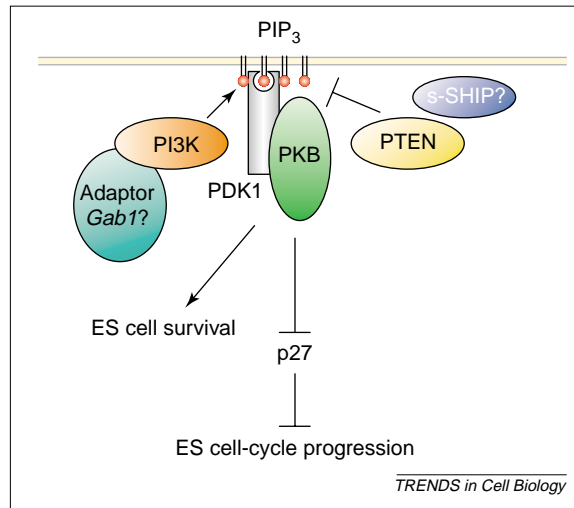


Fig. 3. PI3K-dependent signals promote ES cell survival and cell-cycle progression. Localization of phosphatidylinositol-3-OH kinase (PI3K) at the plasma membrane, through a receptor-associated adaptor protein such as Gab1, increases the amounts of 3'-phosphorylated inositol lipids [PtdIns(3,4,5) P_3]. These molecules colocalize with the phospholipid-binding kinases PDK1 and PKB, and promote the activation of PKB by PDK1. In ES cells, potentiation of PI3K signalling by mutation of the PI3K antagonist PTEN, a 3'-phosphate lipid phosphatase, enhances ES cell survival and increases proliferation rate by suppressing activity of the cell-cycle inhibitor p27^{Kip1}. The report of a novel lipid phosphatase (s-SHIP) expressed in ES cells and haematopoietic stem cells is tantalizing; however, a role for this molecule in stem cell signalling has yet to be established.

which is independent of Ras. But specific attenuation of ERK signalling – either by pharmacological inhibition of MEK activity or by forced expression of ERK phosphatases – also facilitates self-renewal by reducing differentiation ([25] and T. Burdon, unpublished). Notably, inhibition of ERK does not replace the requirement for activation of STAT3 but rather enhances the actions of STAT3, although it is not clear whether this effect is direct or indirect. The indications that ERK activation has a pro-differentiation effect and is antagonistic to ES cell self-renewal are corroborated further by genetic disruption of either *Grb2* [27] or *Shp2* [28], which results in impaired differentiation (Fig. 2). Reintroducing either a *Grb2*-Sos chimaera or an activated form of Ras into *Grb2*^{-/-} ES cells restores normal differentiation [27].

ERK activity reflects the input not only of gp130 cytokines but also of ligands that stimulate Ras through receptor tyrosine kinases and other cell-surface receptors. The overall balance of conflicting activation of STAT3 and ERKs might determine the efficiency of ES self-renewal [29].

PI3K signalling in ES cell propagation

An increased amount of 3'-phosphorylated phosphoinositides is frequently associated with growth factor and cytokine signalling pathways. This increase occurs through receptor-mediated translocation of PI3K to the cell membrane. The PI3K products phosphatidylinositol (3,4)-bisphosphate

[PtdIns(3,4) P_2] and phosphatidylinositol (3,4,5)-trisphosphate [PtdIns(3,4,5) P_3] are ligands or the PH domains of several signal transducers, including the serine/threonine kinases, phosphoinositide-dependent kinase 1 (PDK1) and protein kinase B (PKB)/Akt. Coordinate localization of these lipid-bound kinases at the membrane facilitates PDK1-mediated phosphorylation of PKB, which then modulates the activity of key regulators of apoptosis, the cell cycle and cellular metabolism in various types of cell [30]. The PI3K signalling pathway is also linked to cellular transformation. The tumour suppressor PTEN is a lipid phosphatase that functions as a negative regulator of the PI3K pathway by removing the phosphate from the 3' position of 3-phosphoinositides.

The PI3K signalling pathway has been implicated in ES cell propagation through studies on *Pten*^{-/-} ES cells. These cells have both enhanced viability and an increased rate of cell proliferation [31]. Improved cell survival is correlated with elevated amounts of PtdIns(3,4,5) P_3 enhanced phosphorylation of PKB, and inactivation of the pro-apoptotic protein Bad. An accelerated transit through G1 seems to be caused by an increase in the rate of degradation of the p27^{Kip1} inhibitor of cell-cycle progression (Fig. 3). Notably, the increase in the rate of cell division is more marked in *Pten*^{-/-} ES cells than in *Pten*^{-/-} fibroblasts, which suggests that PI3K-dependent signals could be relatively more significant in regulating the cell cycle of ES cells than in regulating that of fibroblasts.

The PI3K-dependent signals that influence the proliferation and survival of ES cells have not been defined. Although PKB might seem to be a likely candidate, ES cells lacking the upstream activator PDK1 are viable with no reported proliferation defect [32]. They show negligible activation of PKB and also fail to activate other targets of PDK1, including p90 Rsk and p70 S6 kinase. These results raise the possibility that PI3K can influence ES cell growth through a pathway that is not dependent on PDK1/PKB. The PKB-related protein serum and glucocorticoid-induced kinase 1 (SGK) might fulfil this role [33,34]. Alternatively, the PI3K/PDK1 pathway might have a supportive, but dispensable, role in self-renewal.

Unique signalling adaptors in ES cells

Embryonic stem cells express a variant of SH2-containing inositol 5'-phosphatase (SHIP) that lacks the SH2 domain [35]. This enzyme normally removes 5' phosphates from the lipid products of PI3K, and in some systems it inhibits the activation of downstream signals such as PKB. The variant expressed in ES cells is reported to bind the adaptor protein Grb2, but remains unphosphorylated and does not associate with the docking protein Shc [35].

Embryonic stem cells also specifically express large amounts of a variant Gab1 molecule. This protein lacks the N-terminal PH domain, which

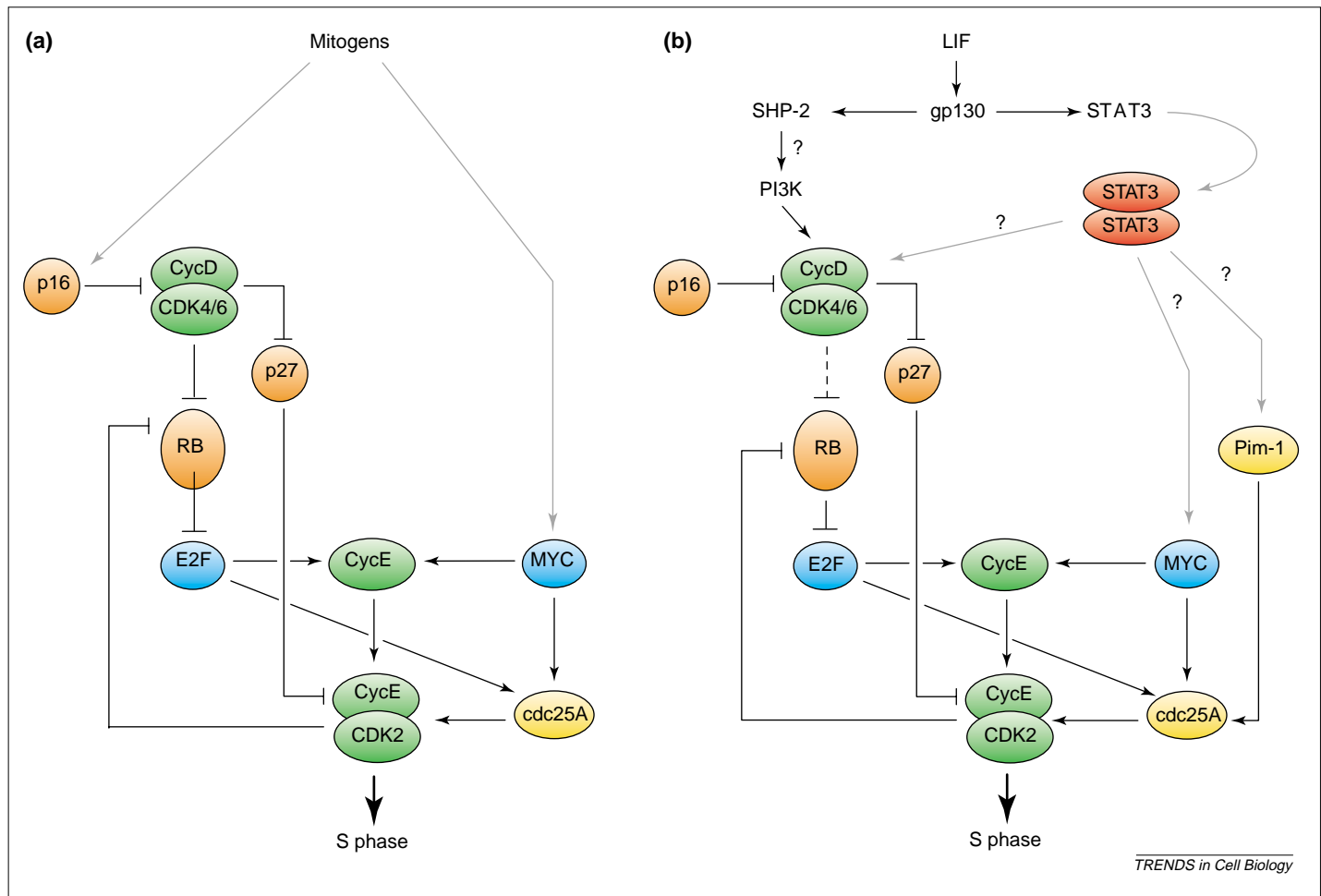


Fig. 4. Models of cell-cycle control in differentiated cells and embryonic stem (ES) cells. (a) The RB/E2F and Myc pathways function in parallel to control accumulation of cyclin E/CDK2 and entry into S phase in differentiated cells. (b) Hypothetical pathways for regulating the accumulation of cyclin E/CDK2 in ES cells. The broken line indicates that cyclin D/CDK4 or cyclin D/CDK6 kinase activity seems to be dispensable in activating RB.

results in attenuated coupling to the Ras/ERK cascade (T. Burdon and A. Smith, unpublished). The presence of these novel isoforms indicates that the signalling circuitry in ES cells is likely to differ from that in other cell types, although the functional significance of these molecules is yet to be determined.

Cell-cycle control differs in differentiated cells and ES cells

Proliferation of differentiated mammalian cells is controlled primarily by regulating the progression through G1 phase and entry into S phase. The retinoblastoma (RB) protein and its relatives p107 and p130 are essential components in the control of the G1/S transition (Fig. 4). The activity of RB is regulated by phosphorylation: hypophosphorylated (G1-specific) RB inhibits the expression of genes that are required for entry into S phase by sequestering the E2F family of transcription factors. During progression through G1, RB is phosphorylated sequentially by complexes of cyclins and cyclin-dependent kinases (CDKs). Phosphorylation by cyclin D/CDK4 or cyclin D/CDK6 induces a partial

release of E2F, which is sufficient to activate transcription of the *cyclin E* and the *cdc25A* genes. The *cdc25A* phosphatase removes inhibitory phosphates from CDK2, and the resulting cyclin E/CDK2 complex then completes RB phosphorylation, leading to full release of E2F, activation of target genes and entry into S phase [36–38].

A second pathway involves the *c-myc* proto-oncogene, which directly stimulates transcription of the genes that encode cyclin E and *cdc25A* to generate cyclin E/CDK2 kinase [38] (Fig. 4). The Myc and the RB/E2F pathways are now thought to be two parallel and cooperative G1/S control pathways that converge on cyclin E/CDK2 kinase – the activity of which determines entry into S phase [38]. Mutations in either or both pathways are frequently encountered in cancers [39,40], reflecting their fundamental role in controlling the cell cycle. The tumour suppressors p16^{ink4a} and p27^{kip1} are inhibitors of cyclin D/CDK4 or cyclin D/CDK6 and cyclin E/CDK2, respectively. They are activated in response to various growth inhibitory signals, including senescence, contact inhibition and terminal differentiation [41].

Embryonic stem cells have a short G1 phase of roughly 1.5 h during which hypophosphorylated RB is virtually undetectable [9]. Thus, RB is likely to be rephosphorylated immediately after mitosis in

ES cells, in contrast to differentiated types of cell. An important issue therefore is whether ES cells are subject to G1 regulation by RB or other members of the RB family, or whether RB is functionally inactivated by constitutive hyperphosphorylation. In addition to RB, ES cells express p107 [42] but not p130 [43]. But much evidence supports the notion that the RB pathway does not regulate the ES cell cycle.

First, ES cells are refractory to the growth inhibitory activity of p16^{ink4a} [10]. Resistance to growth inhibition mediated by p16^{ink4a} is a common feature of cancer cells in which the RB pathway is disrupted [44,45]. Withdrawal of LIF and subsequent differentiation is accompanied by sensitization to growth inhibition mediated by p16^{ink4a}, which indicates that RB control of G1 is imposed during differentiation [10].

Second, inactivating disruptions in all three genes of the RB family [*p107*^{-/-}*p130*^{-/-}*Rb*^{-/-} triple knockout (TKO) cells] do not seem to compromise the proliferation of ES cells but do reduce differentiation in teratocarcinomas [46,47]. This indicates further that RB dependence is acquired only as ES cells undergo differentiation.

Last, ES cells share striking similarities in proliferative behaviour with TKO embryonic fibroblasts. Both TKO MEFs and ES cells fail to arrest in G1 at confluency [46,47]. In normal fibroblasts this phenomenon is accompanied by increased amounts of p27^{kip1}, decreased amounts of cyclin D1 and an accumulation of hypophosphorylated RB, which leads to G1 arrest [48]. ES cells and TKO MEFs also escape replicative senescence and are immortal [46,47]. In other cells, replicative senescence and G1 arrest are associated with an accumulation of hypophosphorylated RB, which is caused by inhibition of CDK kinases mediated by p16^{ink4a} and p21^{cip1} [49]. Both ES cells and TKO MEFs fail to arrest in G1 after DNA damage, but they do arrest at the RB-independent G2/M checkpoint [46,47,50,51]. ES cells, like TKO MEFs, therefore escape from contact inhibition, are immortal and lack the G1 checkpoint. Together, these data strongly support the notion that ES cells are not controlled by RB in G1.

Cyclin expression and function during G1 in ES cells

What mechanism underlies the functional inactivation of RB in ES cells? In certain tumour cells that do not have a mutation in the *RB* gene, RB protein is hyperphosphorylated by constitutive expression of cyclin D/CDK4, cyclin/CDK6 and/or cyclin E/CDK2 kinases [40,52,53].

Cyclin D1 and cyclin D3 are present in low amounts in ES cells, whereas cyclin D2 is not expressed. CDK4-associated kinase activity is virtually undetectable. The low amount of D-type cyclins in ES cells reflects the situation in epiblast cells, which do not express appreciable quantities of D-type cyclins until gastrulation commences [54].

The differentiation of ES or epiblast cells results in robust expression of D-type cyclins and appreciable CDK4-associated kinase activity, which signifies the adoption of G1 regulatory control [10].

Regulation of the basal expression of cyclin D1 differs between ES cells and other cells. First, the Ras/ERK pathway, which is central to transcriptional activation of cyclin D1 expression in somatic cells stimulated by growth factor [55–57], does not contribute to the expression of cyclin D1 in ES cells [58]. Second, the amount of cyclin D1 protein [59] is dependent on PI3K signalling, but this seems to be uncoupled from any specific mitogenic stimulation [58]. Thus, neither PI3K activity nor cyclin D1 expression is downregulated after serum starvation.

Taken together, these data lead us to the conclusion that basal expression of cyclin D1 is disconnected from mitogenic signals transduced by tyrosine kinase receptors in ES cells. Constitutive, albeit low, expression of cyclin D1 could contribute to constitutive phosphorylation of RB. Alternatively, the functional significance of cyclin D/CDK4 complexes in ES cells might be to sequester p27^{kip1} and prevent this inhibitor acting on cyclin E/CDK2 kinase [41]. The resistance to p16^{ink4a} implies, however, that neither function is essential for ES cell proliferation.

In differentiated cells, enforced expression of cyclin E is sufficient to overcome growth arrest that is mediated by p16^{ink4a}. Constitutive cyclin E/CDK2 activity in fibroblasts is also associated with anchorage-independent growth [60], another property shown by ES cells. The *cyclin E* gene is subject to repression by the active form of RB in differentiated cells but, as we have discussed above, the RB pathway seems to be inoperative in ES cells. Consistent with this, an active form of RB is undetectable (L. Vitelli and P. Savatier, unpublished) and cyclin E/CDK2 kinase activity seems to be constitutive in these cells [10].

Gp130 signalling and cell-cycle control in ES cells

The G1/S transition thus seems to be driven uniquely by cyclin E/CDK2 during ES cell self-renewal. A currently unresolved issue is whether the apparently constitutive activity of cyclin E/CDK2 is an intrinsic property of ES cells or is dependent on gp130 signalling.

Withdrawal of LIF induces differentiation of ES cells rather than cell-cycle arrest. But because cell-cycle regulation changes early in differentiation, this does not preclude the possibility that STAT3 could direct the expression of key regulators of the mitotic cycle in ES cells and stimulate their entry into S phase. STAT3 can influence G1/S transition in some types of differentiated cells. In the lymphoid cell line BAF-03, STAT3 activates expression of specific cell-cycle regulators including D-type cyclins, p27^{kip1}, c-Myc and Pim-1 [61].

Pim-1 is a serine/threonine kinase that phosphorylates and activates cdc25A, thereby

potentiating the accumulation of active cyclin E/CDK2 kinase. Myc and Pim-1 combine synergistically to effect interleukin 6 (IL-6)-dependent proliferation of BAF cells [62]. Enforced expression of Myc and Pim-1 is sufficient to overcome cell-cycle arrest mediated by IL-6 starvation, which indicates that they are essential targets of STAT3. This pathway can drive RB hyperphosphorylation and entry into S phase in the presence of minimal cyclin D/CDK4 or cyclin D/CDK6 complexes (Fig. 4b).

Although the lack of a reported proliferation phenotype in ES cells lacking *myc* or *pim-1* seems to argue against such a mechanism operating in ES cells, this lack of phenotype could also be explained by functional redundancy with related members of these gene families. Data on transcriptional activity of effectors of G1/S transition is necessary to determine whether STAT3 directly stimulates proliferation (Fig. 4b) or whether ES cells cycle autonomously until entering into differentiation.

It is also possible that LIFR or gp130 signalling could contribute to G1/S transition by recruiting PI3K through SHP-2 and Gab-1 (Fig. 4b). This pathway has been identified in the T47D breast cancer cell line, in which IL-6 has been shown to control cell migration by activating MAPK and PI3K via the gp130/SHP-2/Gab1 pathway [63]. In ES cells, LIF-dependent activation of PI3K seems to sustain cyclin D1 by positively regulating the rate of its synthesis via p70 S6 kinase and by negatively regulating the GSK3-dependent rate of protein degradation [58]. Observations from ES cells lacking PTEN indicate that PI3K signalling also promotes degradation of the p27^{Kip1} inhibitor [31]. These different actions could result in a low amount of p27^{Kip1} that is effectively sequestered by cyclin D/CDK4 (see above), thereby ensuring that cyclin E/CDK2 remains constitutively active. It should be noted that in the presence of serum and feeders, gp130 signalling might not be the only or even the most significant activator of PI3K in cultures of ES cells.

Concluding remarks

As we have discussed above, ES cells have an unorthodox cell cycle in which the G1 control pathways that operate in other types of cell are reduced or absent. Such features are associated with the deregulated proliferation of tumour cells; however, constitutive replication is also a common aspect of early embryo development in many species. This might simply reflect the fundamental requirement of establishing sufficient cell numbers to initiate gastrulation. It is possible, however, that the uncoupling from G1 regulation might also be involved in sustaining the undifferentiated state.

Active hypophosphorylated RB forms complexes with and promotes the activity of differentiation-promoting transcription factors such as MyoD, myogenin and C/EBP [64–67]. Efficient hyperphosphorylation and inactivation of RB and its other family members might therefore be important to shield pluripotent cells from activities that induce differentiation. The acquisition of G1/S regulation, which involves activation of RB, seems to be an early event in the differentiation of ES cells. Another possibility is that constitutive transit through G1 could constrain the temporal opportunity for both chromatin remodelling and the establishment of heritable transcription programs. Imposition of G1 control might be necessary for the heritable changes in gene expression that signify cell commitment. If this were so, then it might explain why ES cells express RB and components of the Ras/ERK pathway: their presence would render ES cells poised to implement G1 regulation immediately on withdrawal of the self-renewal stimulus.

If the cell-cycle properties of ES cells are functionally important for pluripotency, then they should be common to pluripotent human and stem cells derived from non-human primate embryos [8,68,69]. It will be instructive to examine whether this is the case, particularly in light of reports that these cells differ from mouse ES cells in other respects – most notably their responsiveness to LIF [70].

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